

## Metagenomic Profiling of the Bacterial Community Changes from Koji to Mash Stage in the Brewing of Soy Sauce

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### Abstract

The improvement of soy sauce fermentation is restricted by the insufficient information on bacterial community. In this study, bacterial communities in the koji and mash stage were compared based on next-generation sequencing technology. A total of 29 genera were identified in the koji stage, while 34 in the mash stage. After koji stage, 7 genera disappeared and 12 new genera appeared in the mash stage. The dominant bacteria were *Kurthia*, *Weissella* and *Staphylococcus* in the koji stage and *Staphylococcus*, *Kurthia*, *Enterococcus* and *Leuconostoc* in the mash stage. The results provided insights into the microbial communities involved in soy sauce fermentation.

**Key words:** metagenomics, microbial community, next-generation sequencing, soy sauce fermentation

Soy sauce, a traditional condiment popular in East Asia, is fermented with a mixture of soybean, wheat and bran (Tanasupawat *et al.*, 2002). Soy sauce manufacturing is based on natural inoculation and mixed fermentation under an open environment; thus, its microbial community structure is complex and has significant diversity. It is important to identify which microbes are essential for soy sauce brewing. However, only a few dominant microbes have been identified by the culture-dependent analysis method and the culture-independent method based on DGGE. A more comprehensive understanding of the efficient microbes in the different fermentation stages is necessary to improve soy sauce fermentation.

The fermentation of soy sauce involves two fermentation stages: koji fermentation and mash fermentation. It is the addition of brine that triggers the transition from the koji to the mash stage. Addition of brine is crucial to produce the typical flavour of soy sauce. Brine changes microbial growth conditions and therefore affects the microbial community. There were less fungal species in the microbial community, and their composition and changes are relatively clear. However, insufficient information was supplied on the differences in bacterial communities between the koji and mash stage, restricting the improvement of soy sauce production.

Most studies on the microbial components of soy sauce fermentation used cultivation methods (Ito and Dou, 1994; Tanaka *et al.*, 2012; Tanasupawat *et al.*, 2002). These methods do not have the capacity to reveal all microbial communities involved in soy sauce fermentation. Many microbes in the natural environment are non-cultivable by current isolation and culture methods (Mamlouk *et al.*, 2011; Torsvik and Øvreås, 2002). Metagenomics, which does not depend on microbial isolation and culture, can be used to investigate microbial community. Metagenomics has been widely used to study fermented food, activated sludge, water bacteria and rumen (Liaw *et al.*, 2010; Kakizaki *et al.*, 2012; Dai *et al.*, 2012). To date, no metagenomic study has focused on the microbial community of soy sauce.

Soy sauce koji and mash samples were collected from the soy sauce factory (30°04' 01"N, 120°35'54"E) in Shaoxing. They were sampled at the end points of their fermentation stages. The soy sauce was fermented according to the method described by Röling with modifications (Röling *et al.*, 1996). Fermentation was carried out as follows. Defatted yellow soybean was boiled and then cooled to room temperature (RT, approximately 30°C), and wheat was roasted and then cooled to RT before being ground. A mixture of steamed defatted yellow soybean and ground wheat

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(V/V, 5.5:4.5) was inoculated with *Aspergillus oryzae* and kept at 35°C for 42 h to form soy sauce koji. Koji was mixed with 2.5 times the volume of salted water (18 Be' to 20° Be') in a fermentation tank and held at RT for 6 months to produce soy sauce mash.

Five parallel samples were collected from five batches of soy sauce fermentation at the end of the koji and mash stages respectively. The samples from the koji or mash stage were mixed for metagenomics analysis. DNA was isolated from the frozen samples within 3 weeks of collection. Metagenomic DNA was extracted by direct DNA extraction techniques following the method described by Ni (Ni *et al.*, 2010). With the metagenomic DNA from the koji or mash stage as templates, V3 regions of the 16S rRNA gene were amplified using universal primers V3-F (5'-CTACGGGAGGCAGCAG-3') and V3-R (5'-ATTACCGCGGCTGCTGG-3') (Ovreås *et al.*, 1997). The amplified V3 regions of the 16S rRNA gene were purified using a DNA Purification Kit (Tiangen Biotechnology, China). The purified PCR products

were sequenced by Beijing Genomics Institute (BGI) using HiSeq 2000 (Illumina, USA). Mothur software was used to analyse the sequences. Each sequence was assigned in comparison to sequences in the EzTaxon-extended database using BLASTN searches and pairwise similarity comparisons (Chun *et al.*, 2007). Bacterial species were identified following the approach described by Huse (Huse *et al.*, 2008). Sequences that showed more than 97% similarity were regarded as the same operational taxonomy unit (OTU) (Altschul *et al.*, 1997). Bacterial community structure was analysed using OTU-based approaches.

The purity and yield of metagenomic DNA extracted from the koji and mash were assessed according to absorbance ratios. The  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  values of the koji were 1.81 and 1.98, respectively, whereas those of the mash were 1.80 and 2.12, respectively. These results indicate ideal DNA purity. The yields of the koji and mash stages were 0.26 and 0.06 µg DNA/g, respectively, indicating that the biomass of the former

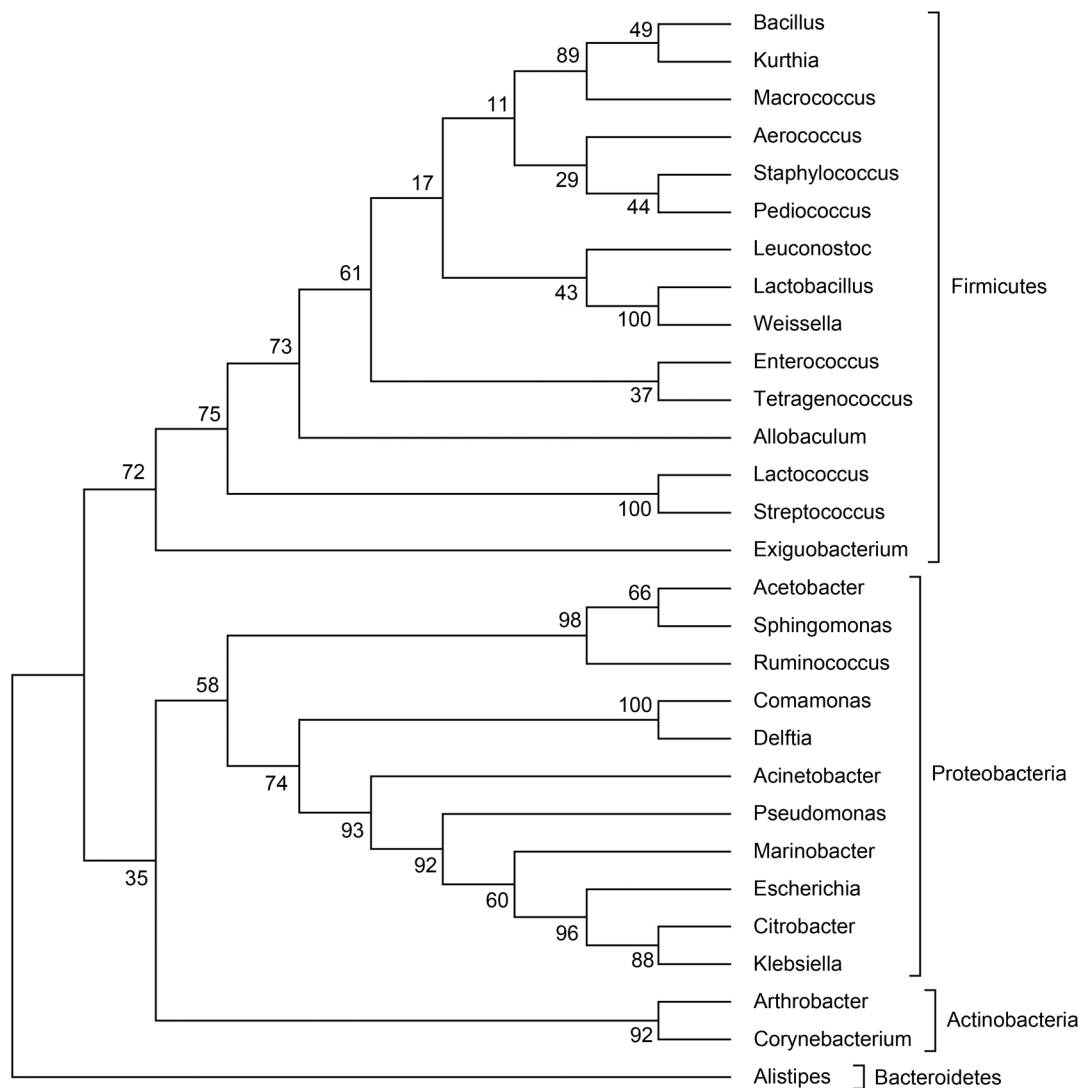


Fig. 1. Neighbor-joining tree of the identified 120 OTUs from the koji

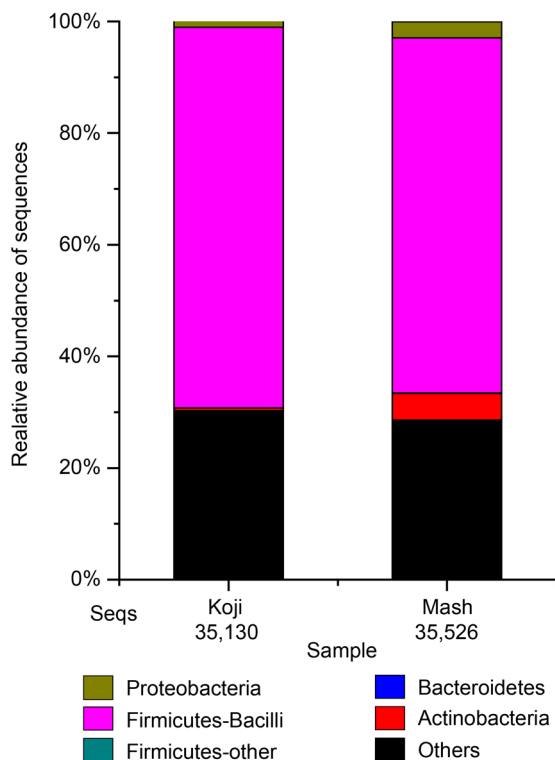


Fig. 2. Relative abundance of sequences from the koji and mash samples

was greater than that of the latter. As expected, intense bands of agarose gel electrophoresis of amplified V3 regions of 16S rDNA were approximately at 200 bp. Next-generation sequencing yielded 70,656 V3 region-trimmed sequences, of which 35,130 were from the koji and 35,526 were from the mash. Their lengths were distributed mainly at approximately 160 bp, and none was shorter than 106 bp or longer than 230 bp. The results were consistent with the known length of bacterial V3 regions (Whiteley *et al.*, 2012).

The OTUs generated by statistical analysis were used to identify species and generate a microbial diversity profile. A total of 181 and 224 OTUs were generated from the koji and mash, respectively. After matching with the database, 0.15% of the OTUs from the koji were mapped at the species level, 67.4% at the genus level and 32.4% were found undescribed in the data-

base. Similarly, 0.22% of the OTUs from the mash were mapped at the species level, 62.1% at the genus level and 37.7% were found undescribed in the database. As shown in Fig. 1, most bacterial in the koji belonged to Firmicutes (103 OTUs, 23,929 sequences, 68.1% of the 35,130 sequences in total); the minority belonged to Proteobacteria (13 OTUs, 343 sequences, 1% of the total sequences), Actinobacteria and Bacteroidetes (4 OTUs, 101 sequences, 0.3% of the total sequences). Within Firmicutes, 101 OTUs (68.1% of the total sequences) belonged to bacilli. As shown in Fig. 2, the proportion of the V3 regions of 16S rRNA gene sequences belonging to bacilli (Firmicutes) were high for both koji (68.1% of the 35,130 sequences in total) and mash (63.42% of the 35,526 sequences in total). The results indicated that most bacteria in the mash came from the koji.

Genus-level identification for both koji and mash is shown in Fig. 3. The relative abundance of each genus in the entire bacterial community is also given. The relative abundance of each genus was calculated as the percentage of its matched sequences from the total sequences. Subsequently, 29 and 34 genera were identified in the koji and mash, respectively. Compared with those in the koji, 7 genera disappeared and 12 genera appeared in the mash. However, these genera only accounted for a very small percentage of the total abundance. The most common genera between the koji and mash were *Kurthia* and *Staphylococcus*, followed by *Weissella* and *Leuconostoc*. The relative abundances of *Acinetobacter*, *Corynebacterium*, *Lactococcus*, *Macrocococcus* and *Streptococcus* increased when the fermentation entered the mash period. Some bacterial genera, such as *Streptomyces*, *Chryseobacterium*, *Paracoccus*, *Aquabacterium*, *Proteus*, *Providencia*, *Salmonella*, *Enhydrobacter*, *Aeromonas* and *Vibrio*, were only detected in the mash.

The microbes that survived in the mash are halotolerant because of the hypertonic environment in the mash (Kapardar *et al.*, 2010). Brine is crucial in the formation of flavour compounds. Its addition promotes the transition from the koji to the mash stage by affecting the microbial community. *Pediococcus*, *Alistipes*, *Allobaculum*, *Arthrobacter*, *Delftia*, *Marinobacter* and

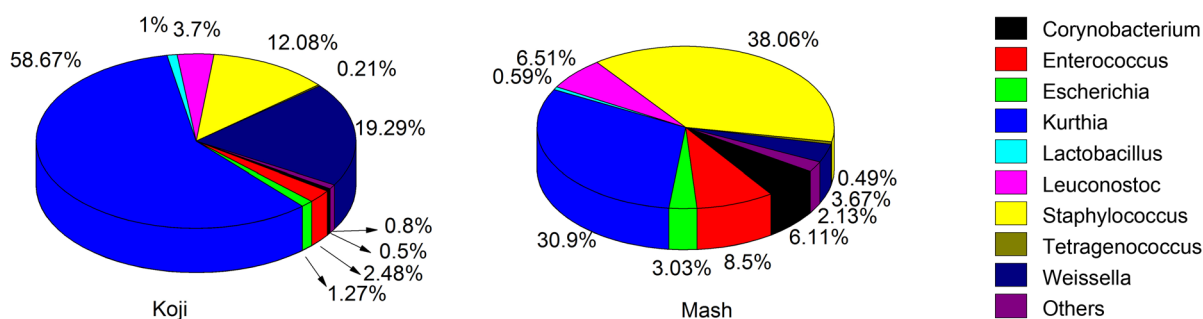


Fig. 3. Genus-level relative abundance and identifications of bacterial community for both koji and mash

*Ruminococcus* were genera not detected in the mash, suggesting that they are restrained by high salt environment and not necessary for mash fermentation. *Kurthia* was the most abundant bacterial genus in the koji. Its relative abundance decreased when the fermentation entered the mash stage. *Kurthia* sp. produces protease and volatile fatty acids in soy sauce fermentation (Steele *et al.*, 1992; Goodfellow *et al.*, 1980). Given that this genus is strictly aerobic, the insufficient dissolved oxygen in the mash restrains its growth. A previous study revealed that *Staphylococcus* sp. helps produce high levels of volatile fatty acids and makes soy sauce show similar sensory characteristics to long-term fermented fish sauce (Wah *et al.*, 2013). *Staphylococcus* sp. is the major bacteria involved in salted and fermented seafood; it has a strong ability to survive in a hypertonic environment (Guan *et al.*, 2011).

In the present study, the metagenomic method showed that *Weissella*, *Staphylococcus* and *Kurthia* were the dominant genera in the koji. *Micrococcus* spp. and *Bacillus* spp. were previously believed to be the predominant bacteria in the koji (Takazane *et al.*, 1998). Wood indicated that genera *Micrococcus*, *Streptococcus*, *Bacillus* and lactic acid bacteria appear spontaneously in the mash brewed by traditional methods (Wood 1985). By contrast, *Micrococcus* sp. was not detected in the mash in the current study. These inconsistent results may be attributed to the differences in specific production technology, fermentation environment or brewing material.

As far as we know, few studies have used metagenomic methods to investigate microbial community changes during soy sauce fermentation. Tanaka *et al.* have investigated the bacterial community in soy sauce using a PCR-DGGE method and found that *Weissella*, *Salmonella*, *Lactobacillus* and *Staphylococcus* genera were dominant in the koji (Tanaka *et al.*, 2012). Our previous study using PCR-DGGE method (Wei *et al.*, 2013), found that *Staphylococcus* sp. and *Bacillus* sp. were the dominant bacteria and detected in the whole fermentation process of soy sauce, while *Kurthia* sp. and *Klebsiella* sp. appeared in the koji fermentation and fade away in the mash fermentation. By comparison, those results based on DGGE method are similar in the aspect of abundant microbes with these obtained in present study based on metagenomic method. Obviously, metagenomic method reached higher species coverage than DGGE-based method, indicating that metagenomic method could detect the microbes with lower abundance.

This study provided new clues to understand soy sauce fermentation process. It was found that 7 genera disappeared and 12 new genera appeared from koji to mash stage. The dominant bacterial genera in the koji stage were *Kurthia*, *Weissella* and *Staphylococcus*, while

those in the mash stage were *Kurthia*, *Enterococcus* and *Leuconostoc*. To the best of our knowledge, this study is the first time to apply metagenomic technology to analyse the bacterial communities involved in soy sauce fermentation. This study will benefit the optimisation of microbial composition and the quality control of fermentation in future research.

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